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identical. Isoproterenol (100 nM) shifted the activation curves nearly identically for both groups, but the ability of isoproterenol to enhance the current was decreased from a 4.1 ± 0.48 fold increase to a 2.64 ± 0.37 ($p < 0.05$). Finally, the recovery rate for calcium current was reduced by hibernation, reflecting an approximate 20 mV shift. These changes in L-type calcium current and isoproterenol response may explain the reduced contractility of hibernating myocytes and the increased the likelihood of sudden cardiac arrhythmias.

## Platform AD: Fluorescence Spectroscopy

**2130-Plat**
**Proton Transfer and Hydrogen-Bond Interactions Determine the Fluorescence Quantum Yield of Bacteriophytochrome, a Novel Deep-Tissue Fluorescent Probe**
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Phytochromes are red-light photoreceptors that regulate a variety of responses and cellular processes. The phytochrome light activation mechanism involves isomerization around the C15=C16 double bond of an open-chain tetrapyrrole chromophore, resulting in a flip of its D-ring. In an important new development, bacteriophytochrome (Bph) has been engineered for use as a fluorescent marker in mammalian tissues (Shu et al. Science 2009). Bph fluoresces at ~720 nm, a wavelength less prone to scattering that can penetrate more deeply into tissue than light emitted by GFP-derived fluorescent proteins. The Bph chromophore biliverdin is a naturally occurring cofactor in mammalian tissue that covalently binds to a conserved cysteine in Bph, and hence BPhs can readily be genetically encoded. BPh photochemistry is thus of considerable significance for biomedical technology. Here we report that an unusual Bph, P3 from Rps. palustris, is highly fluorescent. We identify the factors that determine the fluorescence and isomerization quantum yields of P3 through the application of ultrafast spectroscopy to wild-type and mutants of P3 and a classical Bph, P2. The excited-state lifetime of biliverdin in P3 was significantly longer at 330 - 500 ps than in P2, and accompanied by a significantly reduced isomerization quantum yield. H/D exchange reduces the rate of decay from the biliverdin excite state by a factor of 1.4 and increases the isomerization quantum yield. Comparison of the properties of the P2 and P3 variants in relation to X-ray structures shows that the quantum yields of fluorescence and isomerization are determined by excited-state deprotonation of biliverdin at the pyrrole rings, in competition with hydrogen-bond rupture between the biliverdin D-ring and the apoprotein. This work provides a basis for structure-based conversion of BPh into an efficient near-IR fluorescent marker.

**2131-Plat**
**Fine Tuning the Optical Properties of Green to Red Photoconvertible Fluorescent Proteins**
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Dendra2 is an engineered, monomeric GFP-like protein that belongs to a subclass of fluorescent proteins undergoing irreversible photoconversion from a green- to a red-emitting state upon exposure to purple-blue light. We have measured the X-ray structure of the green species of Dendra2 and performed a comprehensive characterization of the optical absorption and fluorescence properties of the protein in both its green and red forms. The structure, which is very similar to those reported for the closely related proteins EosFP and Kaede, revealed a local structural change next to the chromophore, involving mainly Arg66 and a water molecule. We propose that this structural change explains the blue shift of the absorption and emission bands, as well as the markedly higher pKs of the hydroxyphenyl moiety of the chromophore, which were determined as 7.1 and 7.5 for the green and red species, respectively. The 20-fold enhancement of the neutral species in Dendra2 at physiological pH accounts for the observed higher photoconversion yield of this protein in comparison to EosFP.

**2132-Plat**
**Color Hues in Fluorescent Proteins with the Same Chromophore are due to Internal Quadratic Stark Effect**
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Intrinsically fluorescent proteins (FPs) exhibit broad variations of absorption and emission colors and are available for different imaging applications. The physical cause of the absorption wavelength change in series of mutants with the same chromophore structure, but different surrounding, is however not understood. Here we study the FP series with acylimine-containing red chromophore, in which the absorption maximum varies from 540 nm to 590 nm, and a series of green FPs with phenolate chromophore, where the absorption peak shifts from 450 to 500 nm. We use two-photon absorption spectroscopy to show that the different colors in each series can be explained by quadratic Stark effect due to variations of the strong local electric field within the beta barrel. The model allows us to experimentally access the chromophore parameters, such as vacuum transition frequency ($\nu_0$) and vacuum changes of permanent dipole moment ($\Delta\mu_0$) and polarizability ($\Delta\alpha_0$) upon excitation. Using this purely experimental and all-optical approach, we estimate, for the first time to our knowledge, the amplitudes of the internal electric field (namely its projection on $\Delta\mu_0$) in a protein. These values amount 10 to 100 MV/cm in the mFruits series. Although these fields appear to be very large, they fall well in the range previously estimated theoretically for different other proteins, and are still 1 −2 orders of magnitude smaller than the fields required to ionize the chromophore. Our model brings simplicity to a bewildering diversity of fluorescent protein properties, and it suggests a new way to sense electrical fields in biological systems. On the other hand, it opens up the way to create two-photon brighter FP probes by tuning internal electric field with smart mutagenesis around the chromophore.

**2133-Plat**
**TIRF-Based FRET with One Base-Pair Resolution**
**Seamus J. Holden**, Stephan Uphoff, David Yadin, Johannes Hohlbein, Ludovic Le Reste, Oliver J. Britton, Achillefs N. Kapanidis.
University of Oxford, Oxford, United Kingdom.
Single-molecule FRET (smFRET) is commonly used as a ‘‘nanoscale ruler’’ for the measurement of biomolecular distances and distance changes. However, the limits of FRET resolution for measurements on surface-immobilized molecules have not been rigorously explored. Using total-internal reflection fluorescence (TIRF) microscopy on a set of DNA standards and advanced image analysis software, we have quantified and extended the limits of FRET resolution associated with the use of electron-multiplying CCD (EMCCD) cameras. For such measurements, we derived a novel theoretical description of the major sources of noise ( photon shot noise, background, CCD noise and pixelation effects); we find excellent agreement between our experimental results and predictions from theory and Monte Carlo simulations. For FRET measurements on a truly single-molecule basis (as opposed to measurements on an ensemble of single molecules), analysis of the experimental noise allows us to predict a resolution of 4% FRET within the linear FRET range (20-80%), sufficient to directly observe a distance difference equivalent to one DNA base-pair separation (3.4 Å). For FRET distributions obtained from an ensemble of single molecules (which exhibits broadening due to presence of static heterogeneity), we demonstrate the ability to distinguish between distances differing by as little as 2 base pairs (~7Å). Current work focuses on real-time observation of single-base-pair translocation steps of Escherichia coli RNA polymerase within single early transcription-elongation complexes; such observations are crucial for understanding the mechanisms of DNA and RNA polymerases.
Our work paves the way for ultra-high resolution studies of processes involving conformational changes and protein translocation on nucleic acids.

**2134-Plat**
**Single-Molecule STED with Photostable Fluorophores**
**Robert Kasper**.
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With recent advantage in the development of far-field super-resolution microscopy, further development depends crucially on improved fluorescent probes. We combined STED microscopy with fluorophore stabilization through a reducing and oxidizing system (ROXS) and demonstrate significant improvement of photostability. We show that this improvement can be exploited either for repetitive measurements necessary for 3D or dynamic STED imaging or for resolution enhancement through the application of higher STED beam intensities. Accordingly, a lateral resolution below 30 nm is demonstrated for single organic fluorophores immobilized in aqueous buffer.

**2135-Plat**
**Fluorescence Correlation Spectroscopy Elucidates the Pathway of RNA Interference**
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Short double-stranded RNA molecules have recently emerged as important regulators of gene expression. These small RNAs associate with a member of the Argonaute protein family in an assembly known as RNA-induced silencing complex (RISC).
Here we elucidate the pathway of RNA Interference (RNAi) in vivo by applying fluorescence correlation and cross-correlation spectroscopy (FCS/FCCS). We show that two distinct RISC exist: a large ~3 MDa complex in the cytoplasm and a 20-fold smaller complex in the nucleus. Nuclear RISC, consisting only of Ago2 and a short RNA, is loaded in the cytoplasm and imported into the nucleus. The import of Ago2 into the nucleus is mediated by the import receptor Impotin8.
We further demonstrate that FCCS can be used to study the interaction of different members of the Argonaute protein family with short double-stranded RNAs and their target mRNA molecules.

**2136-Plat**
**G-Quadruplex Folding Observed by two Photon Fluorescence Correlation Spectroscopy and Dual Time Scale**
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G-rich DNA sequences are known to fold upon addition of salt into a stacked well defined configuration called a quadruplex. A fluorescently labeled 5′-FAM −24-mer G-quadruplex sequence was used to explore the variation of diffusion coefficients at extremely low, low and high KCl concentrations. We found a shift in the diffusion coefficient of about 10μm2/sec toward faster diffusion from extremely low to high KCl concentrations. This shift can be related to the compact structure formed by the G-quadruplex. We have also used a fluorescent guanosine analog, 6MI, to label a 24mer that has shown folding behavior at high KCl concentrations. To explore this further, we have added in excess a sequence that complements the G-rich region to deter the formation of the G-quadruplex. The diffusion coefficient also increased from the unfolded, low KCl concentration to the high salt, G-quadruplex structure. We have constructed a dual-timescale (ps TCSPC and uS-mS FCS) photon correlation system and we are using it to explore linked changes in the fluorophores' lifetimes and the translational diffusion coefficients as they move between low and high salt environments. Part of this work was supported by NIH SCORE Grant S06 GM 060654.

**2137-Plat**
**Observing Nuclear Receptor / Coactivator Interactions in Live Cells by Hetero-Species Partition Analysis**
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Measuring the binding curve and stoichiometry of protein complexes in living cells is a prerequisite for quantitative modeling of cellular processes. Dual-color fluorescence fluctuation spectroscopy provides a general framework for detecting protein interactions. However, quantitative characterization of protein hetero-interactions remains a difficult task. To address this challenge we introduce hetero-species partition (HSP) analysis for measuring protein hetero-interactions of the type D + nA -> $DA_n$. HSP directly identifies the hetero-interacting species from the sample mixture and determines the binding curve and stoichiometry in the cellular environment. The method is applied to measure the ligand-dependent binding curve of the nuclear receptor retinoic X receptor to the coactivator transcription intermediate factor 2. The binding stoichiometry of this protein system has not been directly measured yet. A previous study using protein fragments observed a higher binding stoichiometry than biologically expected. We address this difference in stoichiometry by measuring the binding curves of the full-length proteins in living cells. This study provides proof-of-principle experiments that illustrate the potential of HSP as a general and robust analysis tool for the quantitative characterization of protein hetero-interactions in living cells.

# Platform AE: Muscle Regulation

**2138-Plat**
**Determining Mechanism of Phosphorylation of Smooth Muscle Myosin by Calmodulin-Myosin Light Chain Kinase Using an in vitro Model System**
**Feng Hong**, Brain D. Haldeman, Shaowei Ni, Nick Ruana, Del R. Jackson, Josh E. Baker, Christine R. Cremo.
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We have shown that MLCK and calmodulin (CaM) co-purify with unphosphorylated SMM (up-SMM) from chicken gizzard, suggesting that they are tightly bound. Although the MLCK:SMM molar ratio in SMM preparations was well below stoichiometric (1:73 ± 9), the ratio was ~ 23-37% of that in gizzard tissue. Fifteen to 30% of MLCK was associated with CaM at ~1 nM free [$Ca^{2+}$]. There were two MLCK pools that bound up-SMM with Kd ~10 μM and 0.2 μM and phosphorylated SMM with a Kd ~ 20 μM and 0.2 μM. Using motility assays, co-sedimentation assays, and on-coverslip ELISA assays, we provide strong evidence that most of the MLCK is bound directly to SMM through the telokin domain. The bound MLCK can phosphorylate SMM in a $Ca^{2+}$-dependent manner with a $pCa_{50}$ ~ 6 as measured by in vitro motility, similar to in vivo results. After activation of SMM-bound MLCK/CaM with $Ca^{2+}$ and ATP, both motility (0.5 μm/sec) and phosphorylation (>15%) of SMM reach a maximum after ~15-30 min, inconsistent with a free diffusion mechanism. Actin movement over the SMM is not required for this phosphorylation process. Experiments are underway to test the idea that SMM heads proximal to the MLCK-SMM become phosphorylated by a tethered diffusion mechanism.

**2139-Plat**
**The Crystal Structure of the N-terminal 15 Heptads of Smooth Muscle Myosin Rod Offers Insights into the Inhibited State of Myosin**
**Usha B. Nair**, Patricia M. Fagnant, Susan Lowey, Mark A. Rould, Kathleen M. Trybus.
University of Vermont, Burlington, VT, USA.
The coiled coil rod of smooth muscle myosin is important both for regulation of activity and optimal mechanical performance. Myosin with a phosphorylated light chain is active, while in the inhibited, dephosphorylated state the two heads form an asymmetric intramolecular interaction. The minimal myosin that can attain an ''off'' state has two heads and 15 heptads of coiled coil rod, a length approximately equal to that of the myosin head. This observation implies that there may be head-rod interactions in the inhibited state. Here we have determined the crystal structure of this region of the rod. Despite being a parallel, coiled coil dimer, the core arrangement is asymmetric. We propose that this asymmetry is wired into its sequence and crucial to its function. The core of the S2 segment is loosely packed in stretches and the two helical segments are locally off-register or staggered relative to one another. Staggered regions are centered on non-canonical core residues. This relative staggering causes three prominent bends in the coiled coil. Significant deviations from two-fold symmetry are observed in our structure, and to a lesser extent in equivalent crystal structures of S2 fragments from cardiac myosin. The larger variations in stagger and bend angles in the rods of smooth versus striated muscle myosins may explain in part why asymmetric head-head interactions are more prevalent in the thick filament regulated myosins.

**2140-Plat**
**Electron Microscopy and Molecular Dynamics on a D137L Mutant of Tropomyosin**
**Jasmine Nirody**[1], Xiaochuan Li[1], Duncan Sousa[1], John Sumida[1], Stefan Fischer[2], Sherwin S. Lehrer[3], William Lehman[1].
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It is generally agreed that constraints on the curvature and flexibility of tropomyosin are necessary both for the binding and regulatory movements of tropomyosin on actin filaments. It follows that mutagenesis of residues that may affect curvature and/or flexibility is commonly used as an analytical tool. The tropomyosin coiled-coil is stabilized by hydrophobic residues in the "a" and "d" positions of its heptad repeat. However, a highly conserved Asp137 places a negative charge on each chain in a position typically occupied by hydrophobic residues. Substituting a canonical Leu for Asp137 suggested that Asp137 destabilizes tropomyosin and imparts flexibility (Sumida et al., 2008). The D137L mutant does retain F-actin binding properties. We have now assessed changes of curvature and flexibility by EM and Molecular Dynamics (MD) on the Leu137 mutant. Contrary to expectation, rotary shadowed D137L tropomyosin is more curved, not straighter, than control tropomyosin. Moreover, overall the average MD shape of the molecule is extremely bent and, unlike wild type tropomyosin, does not match the contours of the F-actin helix at all. We find that the persistence length of D137L is half that of wild-type tropomyosin (measured either on EM images or on MD frames), indicating that the mutant is more curved and more flexible than the wild type is. MD shows that there is a modest decrease in curvature in the surrounds of residue 137 in the D137L mutant, but it is accompanied by a large unexpected increase in curvature near residue 175. Thus we find that mutation at one site on tropomyosin leads to an unexpected delocalized change at another site along the molecule.